STUDIES IN THE PHYSIOLOGY OF COPRINUS

INAUGURAL DISSERTATION

BY

LISBETH FRIES

BOHUSLÄNINGENS AB - UDDEVALLA 1956

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BY DUE PERMISSION OF THE PHILOSOPHICAL FACULTY OF THE UNIVERSITY OF UPPSALA, TO BE PUBLICLY DEFENDED IN THE BOTANICAL AUDITORIUM, APRIL 28, 1956, AT 10 A.M. FOR THE DEGREE OF DOCTOR OF PHILOSOPHY. THE ARTICLES I—V SUMMARIZED IN THIS SURVEY WILL ALSO BE PUBLICLY DISCUSSED.

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Introduction

This paper summarizes the main results of five earlier published articles dealing with the physiology of the hymenomycete genus *Coprinus*. As appears from the titles of these articles the physiology of the *Coprinus* species has been investigated from various points of view:

I. Studies in the physiology of *Coprinus*. I. Growth substance, nitrogen and carbon requirements (FRIES 1955).

II. Studies in the physiology of *Coprinus*. II. Influence of pH, metal factors and temperature (FRIES 1956 a).

III. Studies in the physiology of *Coprinus*. III. Cultivation experiments with running media (FRIES 1956 b).

IV. Mutations induced in *Coprinus fimetarius* (L.) by nitrogen mustard (FRIES 1948).

V. Factors promoting growth of *Coprinus fimetarius* (L.) under high temperature conditions (FRIES 1953).

These papers will be referred to by the Roman numerals I—V.

The genus *Coprinus* has earlier been the subject of investigations into the physiology of spore germination (e. g. BADEN 1915) and fruit-body production (LAKON 1907, BULLER 1924, BILLE-HANSEN 1953). Careful studies of the sexuality of different *Coprinus* species have been performed by BENSAUDE (1918), MOUNCE (1921), BRUNSWIK (1924) and others, but investigations into the physiology of nutrition are very few. REGE (1927) investigated the ability of a *Coprinus* species to decompose "cellulosic materials", and WAKSMAN (1931) established that *C. radians* was a good decomposer of hemicellulose and cellulose. LEONIAN and LILLY (1938, 1940) found that *C. lagopus* and *C. tergiversans* responded to certain growth factors. JOHNSON and JONES (1941) investigated the influence of temperature and pH on the growth of *C. cubensis*. VORDERBERG (1948) studied the growth conditions of *C. lagopus* but used the fruit-body production as the only measure of the suitability of different carbon sources, nitrogen sources, etc., as did BILLE-HANSEN (1953) in his investigations of other species. Some data about the influence of vitamins, different pH values and metals on the growth of certain *Coprinus* species have been published in a preliminary report (FRIES 1945).

I. Growth substances (I, IV, V)

All tested *Coprinus* species are able to produce good growth in a simple glucose-mineral-salt medium with an addition of only thiamine. Several degrees of thiamine heterotrophy, however, can be noted:

1. Complete inability to synthesize the thiamine molecule, even if the components pyrimidine and thiazole are given: *C. curtus.* This type has earlier been represented by *Phytophthora cinnamomi* (SCHOPFER 1940), but is not yet established among the basidiomycetes.

2. Ability to synthesize the thiamine molecule if pyrimidine and thiazole are given, e. g. *C. cordisporus, C. ephemeroides,* and *C. 24.* This is a very common type among the basidiomycetes.

3. Ability to synthesize the thiazole component of thiamine if pyrimidine as added, and to produce as good a growth with this addition as with thiamine, e. g. *C. ephemerus, C. fimetarius, C. micaceus, C. plicatilis, C. radiatus, C. 20, C. 22, C. 23,* and *C. tardus.*

None of the investigated species is able to synthesize the pyrimidine component if only thiazole is given.

4. Ability to synthesize the thiazole component of thiamine, if pyrimidine is added, but far better growth with pyrimidine + thiazole, e. g. *C. atramentarius, C. comatus, C. hiascens, C. narcoticus, C. niveus,* and *C. picaceus.*

It is noteworthy that in some cases better growth was obtained with pyrimidine+thiazole than with the whole thiamine molecule, e. g. *C. ephemeroides, C. 24,* and *C. hiascens. Boletus luteus* seems to represent the same type (MELIN and NORKRANS 1942). The effect may perhaps be connected with the fact that the thiamine molecule is too big to pass rapidly through the cell membrane.

For most of the investigated species additions of amino acids, vitamins or other compounds present in casein hydrolysate or yeast extract had no growth-promoting effect greater than that of thiamine. The growth of some species was, however, increased to a certain degree, e. g. *C. comatus, C. Friesii, C. picaceus, C. tardus,* and *C. 21.* Additions of enzyme hydrolysed casein (NZ-case) had the greatest effect on the growth of *C. narcoticus,* giving yields four to six times as great as those with thiamine. The effect could not be attributed to any single amino acid or vitamin. Possibly the effect is the result of a specific combination of certain amino acids and/or vitamins, or it depends upon an unknown component of NZ-case.

A dependence of the demand for vitamins or amino acids upon temperature has been observed in bacteria and fungi (e. g. BARNETT and LILLY 1948). Of particular interest is EMERSON's observation (1950) that a strain of *Neurospora* "responds to choline and methionine much better at 35° C than at 25° C." For most *Coprinus* species the demand for growth-promoting substances does not change with the temperature, but in a strain (*III*) of *C. fimetarius,* having its growth optimum at 35°—40° C, the growth rate decreased by additions of casein hydrolysate or yeast extract at 35° C. At 40° C these substances had no effect but at 44° C, near the maximal point for growth, they increased the mycelial production two to six times. The active principle was identified as methionine, which produced its optimal effect in a concentration of 80—240 μmol per litre. Obviously, the biosynthesis of methionine or some metabolically related compound was limiting the growth at +44° C.

The biosynthesis of the sulfur-containing amino acids is rather easily disturbed in bacteria and in fungi. Among the mutants of *C. fimetarius* induced by nitrogen mustard (IV) one parathiotrophic mutant was found, which was later identified as methionine-less. In *C. fimetarius III* the growth at 44° C could also be promoted by homocysteine, but cystathionine and allocystathionine were inactive. The activity of homocysteine, however, was only one tenth of that of methionine, a difference perhaps being due to some insufficiency in the mechanism transforming homocysteine into methionine. None of the possible methyl donors, as choline, betaine and creatine, acted as a limiting factor, since additions of these substances did not enhance the effect of homocysteine, nor did additions of vitamin B_{12}, p-aminobenzoic acid or folic acid.

Other limiting factors to be considered in the synthesis of methionine are homoserine and threonine (TEAS, HOROWITZ and FLING 1948). Threonine alone has no effect, and homoserine has unfortunately not been available for testing. It is of course also a possibility that one of the enzyme systems acting in the formation of methionine is disturbed.

A nutrient solution in which the fungus has grown for at least ten days at 44° C also promotes further growth at this temperature. It does not contain methionine or homocysteine, but a hitherto unidentified factor called HT. This substance is soluble in butyl alcohol, insoluble in ethyl ether, positively charged in acid solution, and evidently labile, since its activity completely disappears in a few weeks. The rapid decrease of the activity by dilution does not indicate the HT factor as an enzyme. An amino acid ought to give a stronger ninhydrin reaction than is produced by this substance. The high growth rate which can be obtained with methionine may be further increased by additions of the HT factor. This fact makes it more likely that methionine is a precursor to the HT factor, than that the HT factor is a precursor to methionine.

II. Nitrogen sources (I)

1. Growth on different nitrogen sources

Experiments have been made to find out whether and to what extent certain *Coprinus* species utilize special nitrogen sources. All investigated species are able to grow on ammonium as well as on amino and amido nitrogen, but not all species prefer the same form of nitrogen. Asparagine and ammonium compounds produce the best growth in most species. *C. 24* and *C. hiascens,* however, give the highest yield on aspartic acid, while *C. 21, C. 22,* and *C. Friesii* prefer urea. The fact that the growth on $(NH_4)_2$-tartrate is in some cases far better than that on $(NH_4)_2HPO_4$ or asparagine might be due to its chelating capacity (FRIES 1945) or to other qualities ascribed to dicarboxylic acids (LEONIAN and LILLY 1940 b). Asparagine, however, has given the highest yields not only of several *Coprinus* species but also of many other fungi, according to the results of other workers. This remarkable quality of asparagine can be interpreted in various ways. Firstly, asparagine can easily give rise to ammonia by deamidation and/or deamination. During the former process aspartic acid is formed, whose important role for the transaminations in plants has been known for several years. However, the most recent investigations have also assigned other qualities to asparagine. WAELSCH *et al.* (1950) prepared an enzyme from bacteria which could catalyze the exchange of the amide group of asparagine for amines. Asparagine may also show another form of activity. An enzyme, catalyzing the transamination between asparagine and certain α-keto acids has been isolated by MEISTER *et al.* (1952). This asparagine transaminase has not yet been prepared from plants, but its existence has been presumed by YEMM 1950.

It is remarkable that *C. fimetarius II* and *C. ephemerus* can produce so good a growth on nitrate, and that *C. micaceus* and *C. narcoticus* are able to use nitrate to some extent, considering the fact that nitrate users seem to be less common among the basidiomycetes.

The growth of some *Coprinus* species on asparagine or aspartic acid has been compared with that obtained when $(NH_4)_2$-tartrate combined with one of these compounds was supplied. The effect of $(NH_4)_2$-tartrate is added to that of asparagine or aspartic acid in the case of *C. fimetarius II,* but only to that of asparagine in the case of *C. curtus.* On *C. 24,* which prefers aspartic acid as a nitrogen source, an addition of $(NH_4)_2$-tartrate to aspartic acid acted inhibiting.

In a medium containing various nitrogen sources the possibility should be considered that they will be utilized for different purposes, for example aspartic acid for certain transaminations, aspargine for others and for peptide synthesis, and so on. When only aspartic acid is given to *C. fimetarius,* very little growth occurs,

probably because the fungus lacks the ability of peptide synthesis with aspartic acid as the primary material, an ability which *C. 24* apparently possesses. When ammonium is also added, this compound may be used directly in several reactions, e. g., to replace the amino nitrogen of the aspartic acid when this is utilized in transaminations and to form asparagine for peptide synthesis. TATUM and co-workers (1935) also assign the favourable effect of aspartic acid $+(NH_4)_2$-tartrate on *Lactobacillus* to asparagine formation. The influence of H^+concentration on the ability of some *Coprinus* species to utilize asparagine, aspartic acid, diammonium phosphate, potassium nitrate and potassium nitrite was investigated. In the cases of *C. fimetarius II* and *C. plicatilis II* asparagine gave the widest range of growth and the smallest change of pH. On the other hand, the growth of *C. hiascens* on aspartic acid compared favourably with that on asparagine. On nitrite *C. plicatilis II* would only grow in a neutral medium, while a little more acid solution could be stood by *C. fimetarius II*. As regards the growth on nitrate, *C. fimetarius II* gave the best yield at pH 5 and 6, quite comparable with that obtained on asparagine.

Little is known of how different pH values of the surrounding media influence the manner of attacking the nitrogen compounds. SCHMALFUSS and MOTHES (1930) showed that *Aspergillus niger* in alkaline solution only utilized amido nitrogen. In acid solution the fungus could enzymatically liberate amino nitrogen from aspartic acid and asparagine, the amido nitrogen of asparagine being simultaneously disengaged. It is very likely that different pH values influence not only the deamidation and deamination but also other enzymatical processes, where asparagine, aspartic acid and the other nitrogen sources are involved.

2. Nitrate and its reduction products

As earlier mentioned, some *Coprinus* species are able to utilize nitrate as a nitrogen source. When using nitrate nitrogen, fungi have to reduce nitrate to nitrite and further on to ammonium or some other reduction product, and in this form the nitrogen enters into the metabolism. Nitrite has appeared to be toxic for several fungi in acid solution, and only a few species have been found utilizing it.

As some *Coprinus* species are able to grow in neutral or alkaline media, their response to nitrite as the only nitrogen source seemed worth while investigating. The growth of 18 different strains or species of *Coprinus* on a medium containing nitrite or nitrate was thus compared with that on a medium with asparagine + $(NH_4)_2$-tartrate. All species earlier found as nitrate users could also utilize nitrite quite as well as nitrate, e. g. *C. fimetarius II, C. narcoticus* and *C. micaceus,*

or far better, e. g. *C. ephemerus, C. fimetarius I* and *III,* and *C. plicatilis. C. curtus,* however, produced a good growth on nitrite though nitrate could not be utilized. The growth on nitrite was for all species more or less inferior to that on asparagine + $(NH_4)_2$-tartrate, and an inhibitory effect of nitrite was also shown, especially in acid solution. Hydroxylamine, presumed as a possible intermediate between nitrite and ammonium, could not be utilized and had an inhibiting effect.

3. The reaction of different strains of C. fimetarius to nitrate and nitrite

The different reactions to nitrate and nitrite shown by the three strains of *Coprinus fimetarius* gave rise to the question whether a distinct physiological difference existed in this respect. The response of different monosporous mycelia of *C. fimetarius II* to nitrate and nitrite was therefore tested. In contrast to the dikaryotic parent mycelium there were certain monosporous mycelia unable to utilize either nitrate or nitrite nitrogen. When spreading over agar plates containing nitrate or nitrite as the only nitrogen source, some of these strains developed spots or sectors of dense aerial mycelium in the otherwise extremely thin mycelium. On nitrate agar plates it was evident that a reduction of nitrate took place, as nitrite could be detected round the spots or the sectors. It was presumed that these alterations were due to spontaneous mutations. This interpretation was supported by the fact that mutations affecting physiological characters have been produced in *Coprinus fimetarius* with nitrogen mustard by the author (IV).

The observation that the ability to reduce nitrate or nitrite varies greatly among the monosporous mycelia compels us to conclude that this apparently fundamental physiological property cannot be considered a reliable species character.

III. Carbon sources (I)

A comparative investigation was made with different sorts of carbohydrates. Among the monosaccharides glucose, fructose, mannose and xylose could be utilized by all species, arabinose by none, and galactose in most cases but poorly. *C. micaceus* gave a comparatively good growth even on galactose like *Psalliota bispora* (Treschow 1944) and some *Tricholoma* species (Norkrans 1950). The mycelial yield of some species on xylose or galactose increased when small quantities of "start glucose" (Norkrans 1944) were added. Most species produced a very good growth on the disaccharides maltose and cellobiose. Saccharose appeared to be completely or almost completely unavailable to all species in agreement with the observations of Bille-Hansen (1953 a) but in contradistinction to the

results obtained with most other basidiomycetes. Lactose could not be utilized by any *Coprinus* species either.

The utilization of high-molecular carbon sources seems to vary greatly among the *Coprinus* species. The enzyme tests indicating the production of cellulase and polyphenoloxidase (being an indirect test for lignin) revealed three different types:

I. Capable of decomposing cellulose as well as lignin: *C. fimetarius, C. curtus, C. ephemerus, C. tardus* and (according to WAKSMAN 1931) *C. radians.*

II. Capable of decomposing cellulose but not lignin: *C. 24, C. 25* and *C. narcoticus.*

III. Capable of decomposing lignin but not cellulose: *C. atramentarius* and *C. comatus.*

To judge from the literature, the first type seems to be the most common among the wood or litter decomposers.

There does not seem to be any apparent correlation between the ecological character of the species and the type of decomposer to which it belongs. It can perhaps be maintained that the pronounced coprophilic species are both cellulose and lignin decomposers (type I). Furthermore, the only two species representing type III prefer soil.

It turned out that starch was utilized to a high degree, while inulin was unavailable for all investigated species. Hemicelluloses, here represented by xylan and mannan appeared to be very favourable carbon sources, a fact which REGE (1927) and WAKSMAN (1931) also found for their investigated *Coprinus* species. Hemicelluloses play an important role also for wood-destroying and litter-decomposing fungi, especially as carbon sources at the start of attacking lignin or cellulose (CAMPBELL 1932). Among the investigated *Coprinus* species there are some showing a very weak positive reaction in the enzyme tests: *C. 24* on cellulose, *C. atramentarius* on "lignin" and *C. plicatilis* on both. These species seem to have no chance in competition with other organisms for available cellulose or lignin. Particulary for these fungi hemicellulose, pectin and even starch appeared very favourable, and therefore they seem to represent a new ecological type of decomposer.

Several species could utilize the polysaccharides and pectins to a larger extent than their hydrolysis products, e. g. xylan better than xylose, pectin better than D-galacturonic acid. It is possible that in utilizing xylan the long chains are hydrolysed to shorter parts, which are absorbed, and this partly degraded form is more rapidly utilized than added xylose. A similar explanation may be suggested for utilization of pectins. In this case it is also possible that smaller parts of the polygalacturonic acid chains are taken up and directly decomposed via other stages than D-galacturonic acid.

IV. pH relations (II)

Earlier investigations into the influence of the H^+ concentration upon the growth of *Hymenomycetes* on liquid media have shown that most species prefer an acid or neutral reaction of the solution. In nature the *Coprinus* species grow on manure or soil rich in humus, which may indicate special demands for a less pronounced acid reaction of the environment. JOHNSON and JONES (1941) also found that *Coprinus* cf. *cubensis* could grow at pH 5.9—9.2 on potato agar.

Several species were cultivated on an ordinary medium containing glucose, mineral salts and with different pH values obtained with phosphate buffers (LINDEBERG 1939, 1944). Some species then showed two maxima on the pH curve and between them a depression at pH 7.5—8. A spectrographic analysis of this medium showed that Fe is not traceable at pH >7, Ca is partly precipitated at pH 6.9 and almost completely precipitated at higher pH values. The concentration of Zn ions decreases at 7.4 to increase again at pH 8. At pH 7.4 apparently only small amounts of Ca and Zn, and perhaps traces of Fe, are present in solution, which coincides with the depression in the pH curves. The maximum of the pH curves above 8 seems to be connected with the increase of Zn ions and the concomitant production of dicarboxylic hydroxy acids (FOSTER 1949), which form soluble and stable complexes with metal ions, e. g. iron.

Different ways of preventing the precipitation of Fe, Zn and Ca and making them available to the fungus were tried out with *C. ephemerus* as a test object. The addition of iron as a soluble and stable complex, ferri-citrate + citric acid (HOPKINS 1930), gave enough ferri-ions for good growth at pH >9 if added after separate sterilization. A slight remaining depression in the curve around pH 7.5 could be eliminated by autoclaving $(NH_4)_2$-tartrate together with the basal solution or by adding ammonia or Ca-lactate after separate sterilization.

Iron precipitates are dissolved at pH 7.5 by carboxylic acids, especially their ammonium salts (REED and HAAS 1924). If iron is added as ferri-citrate + citric acid before autoclaving it will be found among the precipitates after this process, and consequently larger amounts of organic acids are required if precipitation is to be prevented. Thus 0.5 g $(NH_4)_2$-tartrate added per litre of solution before autoclaving gave vigorous growth even at pH >7, probably because ferri-ions were made available. As calcium has proved to be essential for good growth and zinc additions also increase the growth of *C. ephemerus*, these metals are apparently made available when ammonia or $(NH_4)_2$-tartrate is added. This is understandable since calcium and zinc phosphates are soluble in solutions of ammonium salts of hydroxy acids.

Several ways have been tried to obtain a good and handy method for preparing nutrient media of different pH values on which the growth curve obtained gives

a true picture of the reaction of the fungus to different H^+concentrations without being influenced by the precipitation of some essential metals. Thus, in Media 2 and 3 the metals are to a great extent chelated with $(NH_4)_2$-tartrate and citric acid. To prevent precipitations still further Fe, Zn, Ca, Mn and Mg as well as the phosphates were added after separate sterilization in pH experiments (Medium A1+B and A2+B). This method gives good results with several species, but in some cases apparently more favourable compounds are formed while autoclaving the metals together with asparagine and $(NH_4)_2$-tartrate. Sterilization through glass filters is certainly the mildest treatment though the danger of infections is greatest with this method.

Ethylenediamine tetracetic acid (EDTA), recommended by SCHATZ and HUTNER (1949) as a chelating agent, also has been tested. At pH values<7 it has a strongly inhibiting effect and in alkaline solutions it decreases the growth of several fungi to some degree, probably because EDTA here makes essential amounts of necessary metals in the media unavailable to the fungus.

In spite of the probable imperfections of the methods used, some conclusions can be drawn from the experiments with different pH values. Such typical coprophilic species as *C. ephemerus, C. 20, C. cordisporus, C. niveus* and *C. tardus,* and presumably also *C. fimetarius I* and *II, C. 22,* and *C. 25* showed optimum growth at pH values about 8. Other species had optimum growth at pH 7, but good growth appeared at pH 8. An optimum at pH 6 characterized only a few species, while *C. micaceus* was the only fungus with the best growth at pH 5. It can thus be stated that the coprophilic *Coprinus* species are extremely basiphilic and even other species of this genus prefer a more alkaline pH range than most other fungi.

V. Influence of metal factors (II)

1. Calcium as a limiting factor for growth

It has several times been shown that calcium has a stimulating effect on the growth of various fungi (e. g. LINDEBERG 1944, STEINBERG 1948), but regarding the absolute necessity opinions have differed. An investigation on the effect of different Ca-supplies on the growth of *C. ephemerus* showed that 1.2—4 mg Ca per litre gave a good and uniform growth. These amounts seem to be bigger than those necessary for green plants (BURSTRÖM 1952). With smaller additions of Ca than 1.2 mg per litre there is no proportion between the dry weight of the mycelium produced and the amount of Ca supplied. The mycelia of these series also grew much more densely and produced daughter colonies. There is a possibility

that the long lag phases in series with small additions of Ca and the irregularity of the growth even in series in quartz flasks without any supply of Ca may be explained as an adaptation of some enzyme systems to use Mg or some other metal as a component instead of Ca.

2. Iron as a limiting factor for growth

The effect of different supplies of iron on the growth of *Coprinus ephemerus* and *C. micaceus* has also been studied. Iron does not seem to exert any quantitative effect. Different amounts of the metal only influence the rate of growth, and traces of iron are enough for vigorous mycelial production even if the rate becomes slow.

VI. Temperature relations (II, V)

Physiological experiments with basidiomycetes are most often performed at 25° C, and this temperature has also been found to give optimum growth for a great number of species (Melin 1925, Humphrey and Siggers 1935, Björkman 1946). The coprophilic *Coprinus* investigated by Rege (1927), however, showed a high optimum, 30°—35° C. About half the 17 *Coprinus* species of the present investigation showed optimum growth at 25° C and had their maximum point at 35° C, or preferred temperatures nearer 30° C for good growth, which ceased completely at 35°—40° C. Only *C. atramentarius* and *C. micaceus* preferred such low temperatures as 15°—25° C and had their maximum point at 30° C. Species growing at higher temperatures are all coprophilic. Thus *C. curtus, C. tardus* and *C. 23* seemed to prefer 30° C or some degree higher for optimum growth and *C. fimetarius,* 35°—40° C. Most remarkable is *C. fimetarius III* giving growth at 44° C, the growth being strongly promoted by a particular nutrient factor (the HT factor, see p. 5 and V).

VII. Cultivation of two Coprinus species on running media (III)

When cultivating fungi in culture flasks, one cannot avoid a number of changes in the medium produced by the activity of the fungus. Therefore, special trans-flowing flasks were constructed for cultivation on running media. To *C. fimetarius II* running Medium A2 + B seemed more favourable during the entire period of

incubation than the standing medium. The trans-flowing flasks were also used for determining the most suitable phosphate concentration of the medium, since it was possible with them to keep a constant pH value without having a great buffering capacity in the medium. A nutrient solution, 0.02 M as regards the phosphates, was the most suitable for *C. ephemerus,* but as high a concentration as 0.08 M could also be tolerated. *C. fimetarius* seemed to prefer 0.02 or 0.005 M solution during the first 4 days, but later a lower concentration proved to be more favourable.

In experiments with varied additions of glucose the same amount, 10 g per litre, turned out to be the most favourable in running as well as in standing liquid, though *C. fimetarius* showed considerably better growth in the trans-flowing flasks. With *C. ephemerus,* on the other hand, the bigger yields were obtained on standing medium. The glucose and nitrogen contents were also varied, but in such a way that a constant C/N ratio always was maintained. A high nitrogen content apparently inhibited the growth rate in standing liquid, and therefore the C/N ratio was especially important when arranging such experiments.

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